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Follicle –Stimulating Hormone Receptor Polymorphisms in Iraqi Women with Primary Amenorrhea

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Abstract

The study was carried to determine Single Nucleotide Polymorphism (rs6165) of Follicle Stimulating Hormone Receptor (FSHR) gene in blood samples of 62 Iraqi women with primary amenorrhea and 40 healthy control females. The research included chromosomal study and serum analysis of 62 patient samples. The samples were collected from Educational laboratories, City of Medicine, Baghdad and Biotechnology Dept. College of Science- Baghdad University through the period from October 2018 to March 2019. The determinations of SNP (rs6165) were carried out by real-time PCR. Results of rs6165 genotyping showed significant variations between PA patients and controls, Inspecting FSHR gene genotypes and allele frequencies in PA patients groups with the control group, revealed that there was significant variation in the heterozygous (AG) and homozygous mutant type(AA) genotype frequencies in (rs6165). the SNP of target gene may have a role in PA patients complaining from idiopathic puberty problems.

Keywords: *primary amenorrhea, Follicle Stimulating Hormone Receptor (FSHR) gene, SNP, Iraqi women patient.*

Introduction

Primary amenorrhea(PA) could be defined as the absence of menses by 14 years of age in the absence of growth or development of secondary sexual characteristics or absence of menses by 16 years of age regardless of the presence of normal growth and development including secondary sexual characteristics and1. According to WHO, amenorrhea stands as a 6th largest major cause of female infertility and affects 25% of women in the reproductive age. During normal female menstruation cycle, Gonadotropin-releasing hormone (GnRH) is released from the hypothalamus, and it works on the pituitary to release FSH and LH and these 2 hormones from the pituitary act on ovaries that finally make estrogen and progesterone to work on the uterus to carry out the follicular and secretory phase of menstrual cycle. Any failure at any level of this normal physiology can cause amenorrhea2. The absence of menses in a female of reproductive age is related to the disturbance of normal hormonal, physiological mechanism or female anatomic abnormalities. The normal physiological mechanism works by balancing hormones and providing feedback between the hypothalamus, pituitary, ovaries, and uterus 3. According to the ministry of health of Iraq statistics for 2017 and 2018, 9.68% and 17.78% of women respectively suffering from primary amenorrhea and problems with menarche.

The FSHR is a 76 kDa G protein-coupled receptor, consisting of 695 amino acids and belonging to the rhodopsin-like receptor subfamily. The FSHR gene is located at chromosome 2.p21, spans more than190K bases and embeds 10 exons and 9 introns. Exons 1-9 encode for the extracellular domain deputed to ligand binding, while the large exon 10 for part of the so-called “hinge region”, for the seven transmembrane-spanning domains and for the intracellular C-terminal tail. FSHR is expressed mainly in granulosa and Sertoli cells, in which it mediates steroid synthesis and gametogenesis. Upon ligand binding, the receptor undergoes a conformational change, leading to the simultaneous activation of multiple signaling pathways at the intracellular level and4. More than two thousand

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SNPs falling within coding and non-coding regions of the FSHR gene were described so far. One of them displaying strong linkage disequilibrium were largely studied in the setting of infertility: p.Thr307Ala (c.919 G > A; rs6165). This SNP is located in the exon 10 of the FSHR gene and leads to an amino acid change in the hinge region protein receptor5. The biggest difference between SNPs and mutations is that SNPs are inherited. Some of these genetic differences have proven to be very important to human health. If those inherited SNPs are high-risk candidates, they deserve our attention, and their investigation will lead to further mechanistic research to develop new treatment programs6.

Patients, materials, and methods patients

This experimental work was carried out in the Educational laboratories, City of Medicine, Baghdad, from October 2018 till February 2019 and at the University of Baghdad, College of Sciences, Department of Biotechnology to investigate molecular parameters. The healthy control group included 40 apparently healthy females of different ages ranged from 12-35 years. Abnormalities were not found in selected blood samples taken from blood donors and therefore being used as controls for comparison with blood samples taken from primary amenorrhea patients. A total number of 62 patients, attended to the Educational laboratories, City of Medicine, Baghdad, were diagnosed with primary amenorrhea. Patient's ages were ranged from 12-35 years. All patients were suffering from primary amenorrhea symptoms. Meanwhile, all blood samples were subjected to molecular studies. Venous blood samples (5ml each) were collected from affected individuals, Six patients were excluded from this study due to chromosomal abnormalities.

FSHR gene SNP

TaqMan fluorescent oligonucleotide probes and primers sequences for FSH receptor gene SNP (rs6165 G>A)designed by[7]which mediates the effects of FSH, is essential for normal spermatogenesis and male reproduction. This study aimed to investigate the effects of the FSHR polymorphisms on idiopathic male infertility and serum FSH levels in Han-Chinese population. Methods: A case-control study was conducted with 364 idiopathic infertile patients (97 nonobstructive azoospermic, 79 oligozoospermic and 188 normozoospermic. And they provided in a lyophilized state by Macrogen Company (Korea), stored at (-23°C). The sequence of probes, forward and reverse primers are listed in the table (1):

Primer Name	**Sequence**	**Annealing temperature**
codon 307-F	5`-TGTCTTCTGCCA GAGAGGATCTC-3	**60°C**
codon 307-R	5`-TCTGAGCTTCATCCAATTTGCA-3	
codon 307-P/T	FAM 5`-CCCTAGtCTGAGTCATA-3	
codon 307-P/C	VIC 5`-CCCCTAGcCTGAGT-3`	

The Genomic DNA was extracted from blood using the Wizard genomic DNA purification kit (Promega, USA) according to the manufacturer instructions, then the samples were subjected to a real-time polymerase chain reaction(qPCR). The reaction mix was adjusted to a final volume of 10 µl as suggested by the manufacturer and included: 5µl GoTaq Probe qPCR Master Mix (Promega/USA), 0.5µl of each primer (each lyophilized primer was dissolved in free nuclease water to prepare the stock solution in a concentration of 100 pmole/µl. Then the working solution is prepared by adding 10µl of the stock solution to 90µl of free nuclease water), 0.5µl of each prob, 1.5 µl DNA, and 1.5µL nuclease-free water. The mix was transferred to a real-time thermocycler (MIC-4 Real-time PCR System, Australia), which was programmed for the following optimized cycles: initial denaturation for 5 min at 95°C (one cycle), 40 cycles of denaturation (20 sec at 95°C), annealing (30 sec at 60°C) and extension (30 sec at 72°C), and finally one cycle of melt curve at 65–90°C.

Table(2): The program for FSH receptor gene SNPs detection

Steps	°C	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	95
Annealing	60	00:30	
Extension	72	00:30	

Statistical Analysis

Data analysis was done by utilizing SPSS for Windows, version 17(SPSS Inc. Chicago, IL, United States). Data appeared as mean ± standard deviation. Shapiro–Wilk normality test was used to determine whether the studied parameters followed a Gaussian distribution. Variables in which the distribution of data did not conform to normality were first log transformed for analysis and then converted back to standard units for presentation. Categorical variables were analyzed by the Chi-square test. Tukey's, Dunnett, and Bonferroni Post Hoc test for multiple comparisons were applied after T-tests.

Hardy-Weinberg equilibrium calculated using a web tool8. The difference in frequencies of genotypes and alleles between the patient groups and the control group were analyzed using the Chi-square test. Odds ratios (ORs) with a 95% confidence interval (CI) were calculated for measuring the strength of the association between the studied genes and primary amenorrhea. The association degrees between variables were analyzed by Pearson correlation analysis. A two-tailed p-value less than 0.05 ($p<0.05$) was considered significant 9.

Results

***FSHR* gene SNP**

The SNP of FSHR gene (G>A rs6165; located on Chromosome2p21-16:242193529 bp) was presented with three genotypes (GG, AG, AA) and two alleles (G and A).

Analysis of Hardy-Weinberg equilibrium (HWE) in (control) group and (patients) group revealed that the genotypes was consistent with the equilibrium, and significant differences ($p <0.01$) were observed between the observed and expected genotype frequencies in control and significant ($p>0.05$) in patients group (Table 3).

Table(3): Number and percentage frequencies of FSHR gene genotypes and their Hardy-Weinberg equilibrium (HWE) in (C) group and (PA) group.

Genotype	Patient (no=55)				Control (no=40)			
	Observed		Expected		Observed		Expected	
	No	%	No	%	No	%	No	%
GG	25	45.4	19.8	36	12	30	15	37.5
AG	16	29	26.4	48	25	62.5	19	47.5
AA	14	25.4	8.8	16	3	7.5	6	15
HWE Analysis	*p*=**0.003** **Significant**				*p*=**0.045** **Significant**			

Inspecting FSHR gene genotypes and Allele Frequencies in (PA) group and (C) group revealed that there was significant variation between these frequencies, Although an increased frequencies of G allele (60 vs. 61%) and a decreased frequencies of A allele (40vs. 38.7 %) were observed in patients compared to controls (Table 4).

In AG Polymorphism, the odds ratio for the AG genotype was 0.25 (0.10 - 0.58)with P value =0.002 indicating that heterozygous genotype AG was a protective factor to (PA) group and mutant type AA which considered as a risk factor for primary amenorrhea according to the odds ratio 4.21 (1.14 - 15.58) with p-value 0.03.

Table (4): Genotype and allele frequencies of the FSHR gene in (C) group and (PA) group.

Genotype No.		patients		control		OR (95.0% CI)	P- value
		%	No.				
GG (Wild type)		25	45.45		30.0	1.94 (0.83 - 4.55)	0.142
GA (heterozygous mutant type)		16	29.1		62.5	0.25 (0.10 - 0.58)	**0.002***
AA (homozygous mutant type)		14	25.45		7.5	4.21 (1.14 - 15.58)	**0.03***
Total		55	100		100		
Allele frequency	***G***	66	60.0		61.3	0.95(0.53-1.71)	0.882
	A	44	40.0		38.7	1.05(0.59 - 1.89	0.882

OR, odd ratio; CI, confidence interval

Discussion

FSHR gene SNP

FSH and its receptor play a major role in the development of follicles and regulation of steroidogenesis in the ovary. The interaction of this hormone with its cell surface receptor initiates a chain of intracellular reactions characteristic of G-protein-coupled receptors ,if any Structural changes occurred in this region that could lead to changes in the amino acid configuration of the FSH receptor gene, resulting in functional changes in the gene in which Some of it leads to enhance functionality of the receptor, while some reduce it10. Many studies investigated about the main cause of amenorrhea and the impact of polymorphisms on the disease, but there is a little knowledge about it in Iraq so this study could help to seek for the main genetic cause of amenorrhea. Our results illustrate that significant differences in the distribution of FSHR genotypes between PA patients and control group. The proportions of Thr/Thr, Thr/Ale and Ale/Ale were 54.54 %, 29.1and 25.45% respectively inpatient group and 12%, 25% and 3% correspondingly in control group, and there was a significant difference between the two. According to11, results showed that There was a difference in distribution between non ovulatory patients and normal-ovulatory controls was significant for polymorphism 6165 (P<0.05 for position307), with a distribution of (AA) and (AG), genotypes higher in patients than controls, that agree with our findings, also there were some studies that disagree with these results and failed to prove the significance of the SNP and disease 12.

Despite the fact that multiple causes might increase susceptibility to primary amenorrhea, the results of this study showed that single nucleotide polymorphisms in FSHR gene might consider as one of the susceptible factors that result in amenorrhea. However, additional investigations are recommended to be directed on other ethnic populations to approve the results of this study.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

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